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Surface modification of macroporous poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) resins for improved *Candida antarctica* lipase B immobilization

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ABSTRACT

Crosslinked macroporous hydrophilic poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) [abbreviated poly(GMA-*co*-EGDMA)] with identical chemical structure (60% of glycidyl methacrylate) but with varied average pore sizes (from 30 to 560 nm), specific surface areas (from 13.2 to 106.0 m^2/g), specific volumes (from 0.755 to 1.191 cm^3/g) and particle sizes (<100 μm–630 μm) were synthesized via suspension polymerization. Modifications of poly(GMA-*co*-EGDMA) with various diamines (1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane), 2-fluoroethylamine, glutaraldehyde and cyanuric chloride were carried out. The influence of the interaction between *Candida antarctica* lipase B (Cal-B) and various carriers during immobilization on the loading and hydrolytic activity (hydrolysis of para-nitrophenyl acetate) of the immobilized Cal-B were studied. Immobilization of Cal-B was performed at different temperatures and pH values. Cal-B immobilized at 30 °C and pH 6.8 was leading to increased activities. Purely physical adsorption between enzyme and copolymer was observed on carriers in which amine or fluorine groups were introduced into the carrier structure by modification with various diamines or 2-fluoroethylamine. As a consequence enzyme loading and activity decreases. In contrary, modification of the poly(GMA-*co*-EGDMA) with glutaraldehyde and cyanuric chloride results in a covalent connection between enzyme and carrier. The obtained results show a significant increase in Cal-B activity. The influence of the amount of glutaraldehyde and cyanuric chloride used for modification was screened. Increasing the amount of glutaraldehyde or cyanuric chloride used for modification resulted in an increase of the enzyme loading. Consequently, higher amount of glutaraldehyde used led to a higher fraction of the enzyme molecules that are covalently connected on to the carrier. As the amount of glutaraldehyde or cyanuric chloride used for modifications increases, activity of immobilized *C. antarctica* lipase B primarily increases, showing the highest value for 0.66% and 0.050% w/w, respectively, and subsequently decreases. We could show that Cal-B immobilized on epoxy-containing copolymer modified with glutaraldehyde and cyanuric chloride performs higher activity than free enzyme powder.

1. Introduction

Enzymes are promising biocatalysts for many organic reactions. They have excellent features like high activity, specificity and selectivity, and can catalyze under mild and environmental friendly conditions [1,2]. However, most of the natural enzymes show none of their profound characteristics in organic solvents and can easily denature under industrial conditions (high temperature, mechanical shear, etc.). Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are generally difficult. Therefore, there have been many studies to stabilize enzyme activity and increase operational stability. Enzyme immobilization is the most important route that can provide enzyme features under industrial conditions. Enzymes have been immobilized on various supports either by physical adsorption, covalent binding or by incorporation in hydrophobic organic–inorganic hybrid materials [3,4]. Enzyme immobilization provides: (i) increased enzyme activity; (ii) increased enantioselectivity, (iii) temperature stability and (iv) easy recovery from the reaction medium for their reuse. On the other hand, immobilized enzymes are protected by solid matrix that limits their conformational variations diminishing unpredictable changes of their characteristic properties. Solid matrix also overshadows enzymes from temperature changes, pH alternation and shaking conditions.

Candida antarctica lipase B is a versatile enzyme for enantio- and regio-selective transformations on many low molar mass and polymer substrates and has been found to possess a broad range

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of catalytic activities for chemical synthesis [5–7]. This enzyme has numerous of other advantages: (i) stability in acidic pH range, (ii) quality of end product, (iii) less side products and (iv) performing at high temperatures. Due to the many benefits of using immobilized enzymes rather than their soluble counterparts, there have been many researchers focusing on the improvement of Cal-B performance using enzyme immobilization. *C. antarctica* lipase B, immobilized onto a macroporous acrylic polymer resin, is a widely used biocatalyst and commercially available (Lewatit VP OC 1600, Bayer; Novozyme 435, Novozymes A/S; Chirazyme, Roche Molecular Biochemicals).

Macroporous copolymers are used frequently for the preparation of various types of ion exchange resins, as inert component for some types of chromatography, as adsorbents, as support for classical catalysts or enzymes in biosynthesis and as membranes for different purposes. Over the last 30 years, Švec et al. [8,9] and Jovanović et al. [10,11] have reported a series of articles relating to macroporous copolymers of glycidyl methacrylate (2,3-epoxypropyl methacrylate), GMA, and ethylene glycol dimethacrylate, EGDMA [abbreviated poly(GMA-*co*-EGDMA)]. The applicability of these copolymers is very much influenced by their porosity. These authors showed that poly(GMA-*co*-EGDMA), in a shape convenient for application, can be obtained by the suspension polymerization when a low-molecular weight inert component is present in the mixture of monomer and initiator (monomer phase). The inert component is usually a mixture of cyclohexanol and an aliphatic alcohol. The amount of the crosslinking agent, EGDMA, in monomer mixture, the type and the amount of inert component in the monomer phase have the largest influence on the porous structure of poly(GMA-*co*-EGDMA) and varying those parameters results in a copolymer with the same chemical composition but with different porosities and particle sizes. Among the other factors, these copolymer beads have excellent features due to the presence of the epoxy groups which can rapidly react with various reagents [12]. With this, the copolymer characteristics can be easily adjusted to the desired applications. Furthermore, functionalization of these copolymers with various diamines opens possibilities for further modification.

The success of the immobilized enzymes is closely related to the properties of the carrier material and the characteristics of the enzyme. The interactions between support and enzyme are critical for the activity of the enzyme. These interactions can change the enzyme three-dimensional structure, which can either lead to inactivation or to hyper activation.

The type of the interactions between enzyme and carrier is crucial which was shown by Bryjak et al. [13]. Modification of the carrier (hydrophobic acrylic carrier crosslinked with ethylene glycol dimethacrylate and diethylene glycol dimethacrylate) with 1,2-diaminoethane and 1,4-diaminobutane resulted in an altered binding type between enzyme and carrier. Physical adsorption gave preparations with high activity and specific activity but poor storage stability, while covalent attachment gave less active but stable preparations.

It was shown by Betancor et al. that by controlling the amino-containing support activation with glutaraldehyde, it is possible to have one or two glutaraldehyde molecules per primary amino group, and obtain carriers that will show different enzyme activity and enzyme stability [14]. Furthermore, D-amino acid oxidase and β-galactosidase immobilized on different glutaraldehyde supports showed a higher activity and stability than soluble enzymes. In our laboratory, we were able to show that *C. antarctica* lipase B immobilized on poly(GMA-*co*-EGDMA) modified with ammonia and subsequently with glutaraldehyde showed a higher activity than immobilized on the starting copolymers.

In this paper, we also show that Cal-B immobilized on poly(GMA-*co*-EGDMA) modified with 1,6-diaminohexane or 1,8-diaminooctane, and subsequently activated with cyanuric chloride performed higher activity then their soluble counterparts. Fahmy et al. observed a similar trend using cyanuric chloride diethylaminoethyl-cellulose ether as a carrier for *Citrullus vulgaris* immobilization [15].

2. Experimental

2.1. Materials

Candida antarctica lipase B (Cal-B) in the form of a dried powder was purchased from Codexis® (Pasadena, CA, USA). Poly(*N*-vinyl pyrrolidone) was purchased from Fluka and all other chemicals were purchased from Sigma–Aldrich and were not purified further.

2.2. Methods

The pore size distributions were determined by mercury porosimetry (Carlo Erba 2000, software Milestone 200). The shape of the beads was observed using a stereomicroscope (Zeiss Stemi SV 11). UV/VIS measurements were performed on a PYE UNICAM SP8-200 UV/VIS spectrophotometer. The copolymer samples were analyzed for their carbon, hydrogen, oxygen and nitrogen content using the Euro EA Elemental Analyzer (EuroVector). Chlorine content was analyzed by Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany).

2.3. Preparation of epoxy carrier

The samples of poly(GMA-*co*-EGDMA) with different porosity parameters were prepared by a radical suspension polymerization as described elsewhere [8,10,11]. In all experiments, 80.5 g of monomer phase, which contained monomer mixture (20.7 g of GMA and 13.8 g of EGDMA), azobisisobytironitrile (AIBN) as an initiator (0.8 g) and 45.2 g of inert component (40.7 g of cyclohexanol and 4.5 g of tetradecanol or hexadecanol for samples SGE-10/14 and SGE-10/16, respectively, and 36.2 g of cyclohexanol and 9.0 g of tetradecanol for sample SGE-20/14) was suspended in 240.0 g of a 1 wt% aqueous solution of poly(*N*-vinyl pyrrolidone), PVP. In the labels of copolymer samples, letter S designates suspension copolymerization, G and E stand for the monomers (GMA and EGDMA). The first number in a sample labels stands for the share of aliphatic alcohol in the inert component (w/w) and the second one for the number of C-atoms in the aliphatic alcohol.

The copolymerization was carried out at 70 °C for 2 h and then at 80 °C for 6 h with a stirring rate of 200 rpm. After completion of the reaction, the copolymer particles were washed with water and ethanol, kept in ethanol for 12 h and then dried in a vacuum oven at 45 °C for 24 h.

The particle size distribution was determined by sieve analysis. The particles with diameter in the range of 630–300, 300–150, 150–100 and <100 μm were used for further investigation.

The shape of the carrier was observed by recording the polymer beads placed onto black surface, using a stereomicroscope (Zeiss Stemi SV 11) in reflection mode at 25x magnification.

The pore size distributions were determined by mercury porosimetry (Carlo Erba 2000, software Milestone 200). The samples were dried at 50 °C for 8 h and degassed at room temperature and a pressure of 0.5 Pa for 2 h. The values of specific pore volume, V_S, and pore diameter that corresponds to half of the pore volume, $d_{V/2}$, were read from cumulative pore distribution curves. The values of specific surface area, S_{Hg}, were calculated on the basis of cylindrical pore model as described in literature [16].

2.4. Modification of epoxy carrier by various diamines

Toluene (25 ml), diamine (10.0, 14.7, 19.3 and 24.0 g of 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane, respectively) and copolymer (0.3 g), were placed in a proper flask and left overnight. The mixture was placed in an oil bath at 70 °C for 8 h, with stirring speed of 150 rpm. After completion of the reaction, aminated particles were removed by filtration, washed several times with ethanol and water and dried at 40 °C in the vacuum oven for 24 h.

2.5. Modification by 2-fluoroethylamine

Copolymer SGE-20/14-d4 (0.3 g) was dispersed in 25 ml of 2-fluoroethylamine solution in DMSO (0.4 g/ml) and left overnight. The mixture was placed in an oil bath at 70 °C for 8 h, with stirring speed of 150 rpm. Copolymer particles were removed by filtration, washed several times with ethanol and water and dried at 40 °C in the vacuum oven for 24 h.

2.6. Modification of epoxy carrier by glutaraldehyde

The modification of epoxy carrier by glutaraldehyde was performed based on the method used by Bilici et al. [17]. This modification includes two steps: amination and glutaraldehyde activation.

Step 1. Amination: Macroporous copolymer particles (0.3 g) were dispersed in an aqueous ammonia solution (20 ml; 25% w/w). The reaction was conducted at 50 °C for 24 h and stirred at 200 rpm. Particles were washed with demineralised water and dried at 40 °C in the vacuum oven.

Step 2. Glutaraldehyde activation: The aminated copolymer particles obtained were dispersed in PBS buffer (pH 8.0, 15 ml) containing 10% (w/w) of glutaraldehyde. The reaction was conducted at room temperature under mechanical stirring for 3 h. The modified copolymer particles were extensively washed with PBS buffer and then dried in vacuum oven at 40 °C.

2.7. Modification of epoxy carrier by cyanuric chloride

The modification of epoxy carrier by cyanuric chloride involves two steps: amination and cyanuric chloride activation.

Step 1. Amination: Macroporous copolymer particles (0.6 g) were dispersed in 1,6-diaminohexane solution in toluene (50 ml; 6.66 M) and left overnight at room temperature. The reaction was conducted at 50 °C for 8 h and stirred at 200 rpm. Aminated particles were washed with demineralised water and then dried at 40 °C in the oven under vacuum.

Step 2. Cyanuric chloride activation: 0.1 g of aminated copolymer particles obtained was introduced into a slurry of cyanuric chloride dissolved in a mixture of 20 ml of dioxane and 4 ml of toluene (0.15% w/w). The mixture was stirred at 200 rpm for 5 h at room temperature. Afterwards, activated particles were intensively washed with toluene and acetone until no more cyanuric chloride was present.

2.8. Enzyme immobilization

The copolymer beads were added to a Cal-B solution (6.67 mg/ml) in PBS buffer pH 6.8. The ratio of copolymer to Cal-B was 4:1 in all experiments. The samples were incubated in a rotary shaker at 200 rpm at 30 °C. After 24 h the solution was removed by filtration and the resulting immobilized Cal-B was washed with PBS buffer and distilled water, until no protein was detectable any more in the washing solution. Supernatant and washing solutions were collected and using the bicinchoninic acid (BCA) protein assay, the amount of enzyme that is immobilized could be estimated. The resulting resins with immobilized Cal-B were freeze dried for 48 h and then used for hydrolytic activity tests.

2.9. Hydrolytic activity

A 1,4-dioxane solution (5 ml) containing *p*-nitrophenyl acetate (*p*NPA) (40 mM) and methanol (80 mM) was added to 20 ml vials containing 0.772 mg of enzyme. The assay reactions were carried out for 50 min at 35 °C (300 rpm) and were terminated by removal of the enzyme by filtration. The concentration of the reaction product *p*-nitrophenol (*p*NP) was determined by UV/VIS spectrophotometer (PYE UNICAM SP8-200 UV/VIS) at λ_{max} (304 nm) of *p*NP. Enzyme hydrolytic activities for immobilized Cal-B is defined herein as the nanomoles of *p*NPA hydrolyzed in 1,4-dioxane per unit of weight of enzyme per time (nmol of *p*NP/min mg).

2.10. Determination of covalent attachment

Immobilized Cal-B (3.0 mg) was added in 1.5 ml of DMSO. The samples were incubated in a rotary shaker at 200 rpm at 30 °C. After 5 h, DMSO, the fraction of the enzyme molecules that are physically adsorbed on the carrier were removed and the resulting covalently attached Cal-B was washed with PBS buffer and distilled water, until no protein was detectable any more in the washing solution. Supernatant and washing solutions were collected and using the bicinchoninic acid (BCA) protein assay, the fraction of Cal-B molecules that are covalently attached to the carrier could be estimated.

3. Results and discussion

It is well known that the amount of bound enzyme and its activity depends on physical and chemical structure of the carrier. Thereby, a series of poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) [abbreviated poly(GMA-*co*-EGDMA)] resins (see Table 1) with identical chemical structure (60% of glycidyl methacrylate) but with varied particle size (<100–630 μm), average pore diameters (from 48 to 270 nm), specific surface area (from 27.6 to 55.2 m^2/g) and specific volumes (from 1.044 to 1.111 cm^3/g) were synthesized and used as carriers for *C. antarctica* lipase B immobilization. These carriers have epoxy groups that are suitable for modification. Hence, these carriers were modified with ammonia and various diamines in order to introduce amino groups to their structure. The presence of amino groups is needed for glutaraldehyde and cyanuric chloride modification. On the other hand, comparison between physically adsorbed and chemically attached enzyme on the carrier plays an important role in this research. All these carriers were studied to assess how these parameters effect Cal-B immobilization and hydrolytic activity (hydrolysis of *p*-nitrophenol acetate).

3.1. Modification with various diamines

Macroporous poly(GMA-*co*-EGDMA) was synthesized in the shape of beads (Fig. 1) by suspension polymerization. Poly(GMA-*co*-EGDMA) is suitable for covalent attachment of Cal-B, since the epoxy group can easily react with enzymes that contains amino group. Determination of covalent attachment (DMSO extraction)

Table 1
Particle size and porosity parameters of poly(GMA-*co*-EGDMA): average pore diameter, specific surface area and specific volume.

Resin	Sample name	Particle size (μm)	Average pore specific diameter[b] (nm)	Specific surface area[b] (m^2/g)	Specific volume[b] (cm^3/g)
1	SGE-10/16	630–300	30 ± 1.2	82.0 ± 4.1	0.923 ± 0.05
2	SGE-10/16	300–150	87 ± 3.5	36.0 ± 1.8	0.755 ± 0.04
3	SGE-20/14	630–300	92 ± 4.6	36.0 ± 1.8	1.111 ± 0.06
4	SGE-20/14	300–150	270 ± 13.5	27.6 ± 1.4	1.040 ± 0.05
5	SGE-20/14	150–100	59 ± 3.0	46.7 ± 2.3	1.080 ± 0.05
6	SGE-20/14	<100	48 ± 2.4	55.2 ± 2.8	1.100 ± 0.06
7	SGE–20/16	630–300	30 ± 1.2	106.0 ± 5.3	1.191 ± 0.06
8	SGE-20/16[a]	300–150	560 ± 28.0	13.2 ± 0.7	1.125 ± 0.06

[a] The particles do not have spherical shape.
[b] Standard deviation values were calculated from three replicate experiments.

was carried out for Cal-B immobilized on resins 1–8 in Table 1. In all the cases, ~80% of Cal-B is covalently attached to the resin.

Resins 1–8 were subsequently modified with various diamines: 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane. By introduction of the amine groups in the carrier structure via modification with various diamines, physical adsorption between enzyme and copolymer will be favored, as covalent attachment via the epoxy group is no longer possible. DMSO leaching experiments showed that 80–88% of Cal-B is physically adsorbed on the aminated copolymer beads.

The elemental analysis data for initial poly(GMA-*co*-EGDMA) (sample 1), samples modified with 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane are presented in Table 2. The results show the presence of nitrogen in the modified copolymers. The theoretical content of epoxy groups in synthesized poly(GMA-*co*-EGDMA) (sample 1), calculated on the basis of the feed composition, was 4.22 mmol/g. The elemental analysis data for the samples are in good agreement with these theoretical values. The difference is within the error limits of suspension polymerization. Detailed studies regarding the reaction of macroporous poly(GMA-co-EGDMA) with primary and secondary diamines were published by Švec et al. [9].

Theoretical degrees of conversion of epoxy groups of poly(GMA-*co*-EGDMA) (resin 1) with 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane are 51.54%, 47.12%, 39.86% and 30.63%, respectively [18]. However, the type of the diamine has pronounced influence on the conversion of the epoxy groups. This could be expected, since the limitation of the reaction due to a steric effect is one of the main problems in polymer functionalization with larger groups [19]. Another limitation in the case of poly(GMA-*co*-EGDMA) is inaccessibility of all epoxy groups for chemical reaction due to high degree of crosslinking. A similar trend was observed for reactions of azole ligands on the sulfur analogue of GMA based macroporous crosslinked copolymers [20].

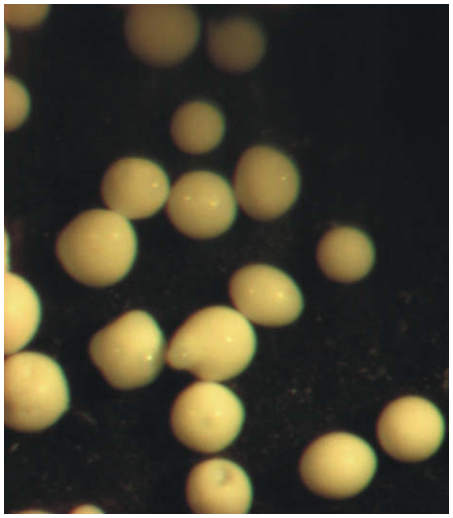

Fig. 1. Microscope image of the poly(GMA-*co*-EGDMA) beads (resin 1).

Table 3 shows the enzyme loading and hydrolytic activity results for Cal-B immobilized on poly(GMA-*co*-EGDMA) and poly(GMA-*co*-EGDMA) modified with various diamines. Enzyme loading is defined as the weight of Cal-B that is immobilized per total weight of carrier. In all samples, the amount of Cal-B adsorbed on the carrier is given as sum of amount of Cal-B physically adsorbed and amount of Cal-B covalently linked to the carrier. In all the cases the enzyme loading is higher for Cal-B immobilized on epoxy-containing copolymer than for Cal-B immobilized on aminated copolymer. These results are attributed to the fact that high fraction of Cal-B is covalently attached to the poly(GMA-*co*-EGDMA) (80%). In contrary, weak physical adsorption is the dominant form of immobilization of Cal-B on the aminated copolymers (from 80% to 88%).

Enzyme activity is not significantly improved by amination of poly(GMA-*co*-EGDMA). Modification of the 630–300 μm beads (resin 1, resin 3 and resin 7) with four various diamines resulted in a slight increase in the enzyme hydrolytic activity. In contrary, modification of the 300–150 (resin 2, resin 4 and resin 8), 150–100 (resin 5) and <100 μm (resin 6) beads, resulted in a decrease of enzyme activity. For instance, Cal-B immobilized on resin 6 (particle size <100 μm) performed much higher hydrolytic activity (4766.0) than immobilized on resin 6 modified with 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane (3142.9, 2143.5, 2243.3 and 2768.1, respectively). These results are attributed to the fact that the amino-functionalization causes considerable alteration of porosity parameters of poly(GMA-*co*-EGDMA) [21,22]. All functionalized samples have higher specific surface and smaller pore size compared to the initial ones. On the other hand, increasing the specific surface area and pore size resulted in a large increase in the enzyme activity [23]. So enzyme hydrolytic activity is influenced by alteration of porosity properties and nature of the binding between enzyme and carrier, caused by amination of the epoxy-containing copolymer.

3.2. Modification with 2-fluoroethylamine

From Table 3 it can be noticed that *C. antarctica* lipase B immobilized on resin 6 modified with 1,2-diaminoethane performed the highest hydrolytic activity. Therefore, resin 6 was modified with 2-fluoroethylamine due to its similar structure with 1,2-diaminoethane, and in order to increase the hydrophobicity of the carrier. By introduction of the fluorine in the carrier structure via modifica-

Table 2
Elemental analysis of initial and amino-functionalized poly(GMA-*co*-EGDMA) samples.

Resin	Elemental analysis[a]					
	Experimental			Calculated		
	%C	%H	%N	%C	%H	%N
Poly(GMA-*co*-EGDMA) (resin 1)	57.57	8.35	–	59.99	7.11	–
Resin 1 modified with 1,2-diaminoethane	53.91	7.67	6.09	56.99	8.05	6.99
Resin 1 modified with 1,2-diaminobutane	55.62	7.86	5.57	58.86	8.47	6.54
Resin 1 modified with 1,2-diaminohexane	58.19	8.24	4.71	60.5	8.83	6.13
Resin 1 modified with 1,2-diaminooctane	58.48	8.16	3.62	61.96	9.15	5.78

[a] Elemental analysis was calculated from multiple determinations within ±0.2% agreement.

Table 3
Cal-B immobilization on poly(GMA-*co*-EGDMA) resins of different particle size, pore diameter, specific surface area and Specific volume and poly(GMA-*co*-EGDMA) resins modified with various diamines: enzyme loading and hydrolytic activity.

Resin	Enzyme loading[a] (μg/mg)	Hydrolytic activity[a] (nmol *p*NP/min mg Cal-B)	Modification	Enzyme loading[a] (μg/mg)	Hydrolytic activity[a] (nmol *p*NP/min mg Cal-B)
1	216.9 ± 3.2	1052.7 ± 22.2	1,2-diaminoethane	80.7 ± 2.0	1894.5 ± 22.9
			1,4-diaminobutane	100.0 ± 3.1	2011.7 ± 31.0
			1,6-diaminohexane	104.5 ± 2.9	2317.9 ± 19.9
			1,8-diaminooctane	103.0 ± 4.5	2599.6 ± 55.9
2	220.8 ± 2.2	2125.0 ± 19.0	1,2-diaminoethane	122.0 ± 5.1	1925.7 ± 88.1
			1,4-diaminobutane	124.6 ± 1.9	2017.1 ± 65.4
			1,6-diaminohexane	116.2 ± 4.4	2177.2 ± 55.9
			1,8-diaminooctane	114.6 ± 3.0	2162.6 ± 29.1
3	185.2 ± 3.6	2110.6 ± 31.2	1,2-diaminoethane	127.8 ± 1.5	2552.5 ± 41.9
			1,4-diaminobutane	117.1 ± 3.3	2680.2 ± 28.2
			1,6-diaminohexane	132.3 ± 2.8	2208.3 ± 33.2
			1,8-diaminooctane	121.3 ± 2.0	1983.3 ± 61.7
4	207.8 ± 4.7	2775.0 ± 69.8	1,2-diaminoethane	109.4 ± 1.8	2316.8 ± 34.5
			1,4-diaminobutane	121.59 ± 3.3	2145.9 ± 20.0
			1,6-diaminohexane	106.4 ± 2.9	2047.5 ± 41.1
			1,8-diaminooctane	116.1 ± 3.0	2291.4 ± 22.8
5	182.7 ± 2.7	4721.0 ± 88.9	1,2-diaminoethane	79.3 ± 4.4	1551.4 ± 77.4
			1,4-diaminobutane	112.9 ± 2.9	1416.0 ± 54.1
			1,6-diaminohexane	113.5 ± 2.5	1990.1 ± 61.0
			1,8-diaminooctane	115.1 ± 1.1	3069.7 ± 41.5
6	151.3 ± 2.8	4766.0 ± 33.6	1,2-diaminoethane	114.0 ± 4.0	3142.9 ± 33.3
			1,4-diaminobutane	102.2 ± 1.7	2143.5 ± 30.0
			1,6-diaminohexane	111.9 ± 2.2	2243.3 ± 28.9
			1,8-diaminooctane	112.6 ± 3.1	2768.1 ± 28.2
7	194.8 ± 1.2	1560.0 ± 19.0	1,2-diaminoethane	105.8 ± 1.4	1955.3 ± 51.0
			1,4-diaminobutane	109.7 ± 2.1	2065.6 ± 34.3
			1,6-diaminohexane	115.7 ± 2.7	1776.1 ± 51.0
			1,8-diaminooctane	108.6 ± 2.9	1924.4 ± 19.9
8	189.2 ± 1.2	3230.6 ± 29.3	1,2-diaminoethane	107.7 ± 1.8	1879.7 ± 22.5
			1,4-diaminobutane	112.1 ± 2.5	1896.3 ± 18.3
			1,6-diaminohexane	104.4 ± 2.1	1778.2 ± 70.9
			1,8-diaminooctane	109.9 ± 1.5	1930.9 ± 66.1

[a] Standard deviation values were calculated from three replicate experiments.

tion with 2-fluoroethylamine, physical adsorption between enzyme and copolymer will be favored. DMSO leaching experiments showed that ~99% of Cal-B is physically adsorbed on fluorinated beads.

The elemental analysis for resin 6 modified with 2-fluoroethylamine showed fluorine content of 3.12% which is in accordance with 38.9% of the conversion of epoxy groups.

Hydrolytic activity tests were carried out and it was found that Cal-B immobilized on fluorinated resin 6 showed a much higher activity (4264.83 nmol *p*NP/min/mg) than free enzyme powder (2396.0 nmol *p*NP/min/mg). Similar behavior was observed with poly(tetrafluoroethylene) particles for α-chymotrypsin immobilization [24]. The process of hydrophobic adsorption offers a simple method for immobilization of enzymes without necessitating chemical manipulations, and with a strong possibility of their native properties remaining unaltered upon binding. By introduction of the fluorine, ability of the carrier for enzyme immobilization is retained and new environment for the reaction system is created.

3.3. Modification with glutaraldehyde and cyanuric chloride

As already mentioned above, epoxy-containing copolymers were modified either with ammonia and subsequently activated with glutaraldehyde [14] or with 4 different diamines: 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane and 1,8-diaminooctane, and subsequently activated with cyanuric chloride. Table 4 shows the enzyme loading and hydrolytic activity results

Table 4
Cal-B Immobilization on poly(GMA-*co*-EGDMA) resins modified with ammonia and activation with glutaraldehyde: enzyme loading and hydrolytic activity.

Resin	Modification	Activation	Enzyme loading[a] (μg/mg)	Hydrolytic activity[a] (nmol *p* NP/min mg Cal-B)
1	ammonia	glutaraldehyde	192.31 ± 2.8	1311.4 ± 39.0
2	ammonia	glutaraldehyde	185.41 ± 2.2	2883.9 ± 32.0
3	ammonia	glutaraldehyde	151.53 ± 4.4	2762.8 ± 40.6
4	ammonia	glutaraldehyde	161.24 ± 2.9	3121.6 ± 32.1
5	ammonia	glutaraldehyde	155.20 ± 3.5	5716.1 ± 21.6
6	ammonia	glutaraldehyde	135.24 ± 3.3	7810.1 ± 28.1
7	ammonia	glutaraldehyde	155.81 ± 8.1	2290.0 ± 62.2
8	ammonia	glutaraldehyde	148.52 ± 5.2	3409.0 ± 55.0

[a] Standard deviation values were calculated from three replicate experiments.

Table 5
Cal-B immobilization on poly(GMA-*co*-EGDMA) resins modified with various diamines and activated with cyanuric chloride: enzyme loading and hydrolytic activity.

Resin	Modification	Activation	Enzyme loading[a] (μg/mg)	Hydrolytic activity[a] (nmol *p* NP/min mg Cal-B)
5	1,2-diaminoethane	cyanuric chloride	79.3 ± 1.2	2193.2 ± 55.0
5	1,4-diaminobutane	cyanuric chloride	112.9 ± 1.4	2075.8 ± 29.0
5	1,6-diaminohexane	cyanuric chloride	113.5 ± 1.8	3231.2 ± 63.2
5	1,8-diaminooctane	cyanuric chloride	115.1 ± 1.2	3186.7 ± 34.3
6	1,2-diaminoethane	cyanuric chloride	114.0 ± 2.2	3321.1 ± 83.3
6	1,4-diaminobutane	cyanuric chloride	102.2 ± 2.1	2605.9 ± 30.1
6	1,6-diaminohexane	cyanuric chloride	111.9 ± 1.3	3808.8 ± 24.7
6	1,8-diaminooctane	cyanuric chloride	106.0 ± 3.1	3130.1 ± 22.2

[a] Standard deviation values were calculated from three replicate experiments.

for *C. antarctica* lipase B immobilized on epoxy-containing resins firstly modified with ammonia and subsequently activated with glutaraldehyde. Results are in agreement with our previous results [23]. Since the highest enzyme activity was obtained using resin 6 as starting material, further work was accomplished with this modified copolymer. Table 5 shows that Cal-B immobilized on resin 6, firstly modified with 1,6-diaminohexane and subsequently activated with cyanuric chloride, performed the highest activity (the rest of the data is not shown since Cal-B immobilized on the copolymers 1–4 and 7–8 primely modified with various diamines and subsequently activated with cyanuric chloride performed significantly lower activity). Thus, further work was carried out with this modified copolymer as a carrier for Cal-B immobilization.

The elemental analysis was performed for resin 6 modified with ammonia and subsequently with glutaraldehyde, and 2.32% of nitrogen was found (indicating successful modification with ammonia) and 32.9% of oxygen was found (elemental analysis of the sample 6 was carried out and 28.9% of oxygen was found), indicating the presence of the glutaraldehyde molecule. The same analysis was carried out for resin 6 modified with 1,6-diaminohexane and subsequently with cyanuric chloride, and 5.85% of chlorine was found, indicating the presence of cyanuric chloride in the copolymer structure.

After modification of resin 6 either with ammonia or with 1,6-diaminohexane, different concentrations of glutaraldehyde and cyanuric chloride were used for surface activation. As can be seen in Fig. 2A, increasing the amount of glutaraldehyde used for mod-

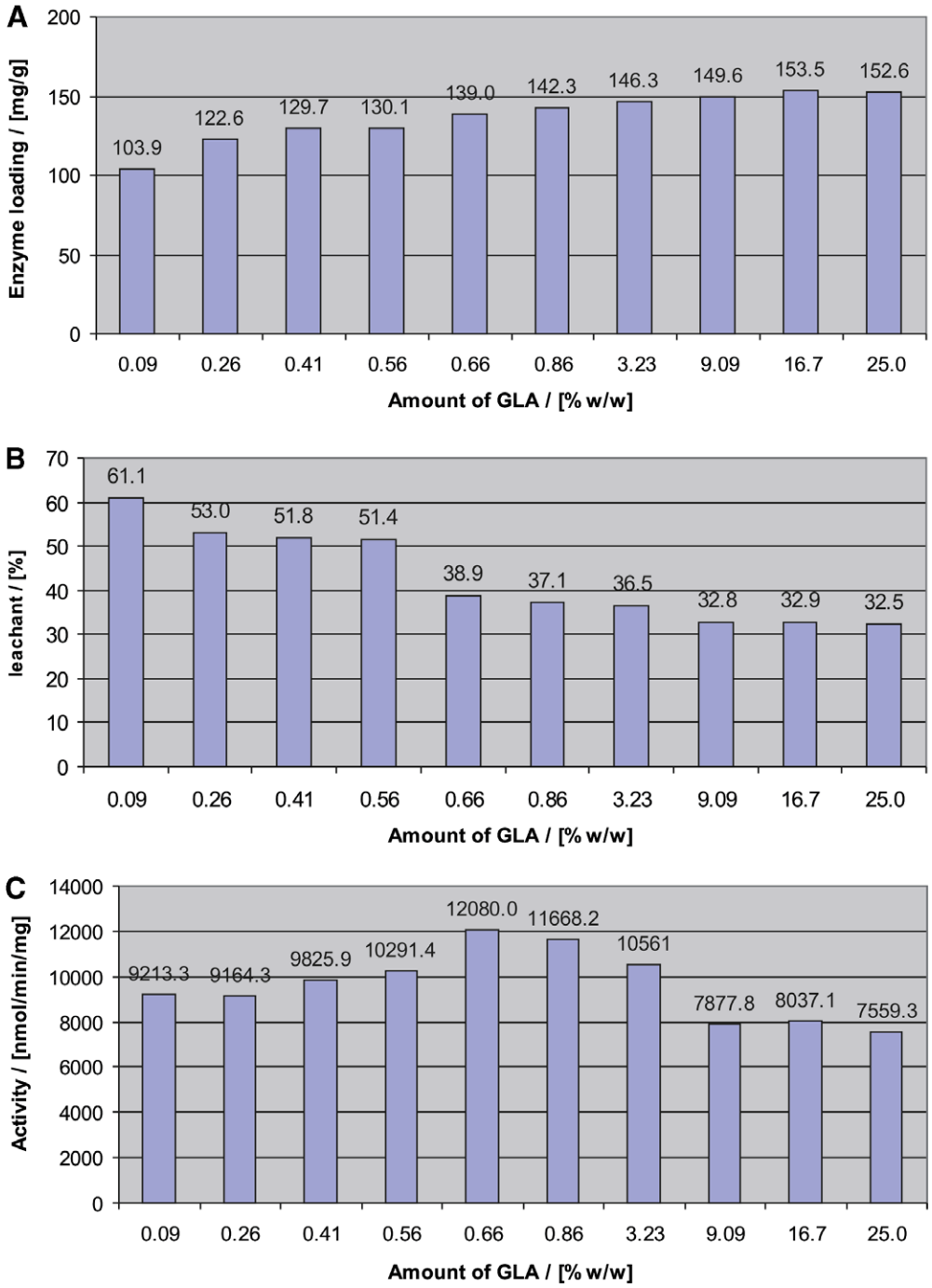

Fig. 2. Influence of the concentration of glutaraldehyde used for activation of aminated copolymer: (a) enzyme loading, (b) leaching and (c) hydrolytic activity.

ification resulted in an increase of enzyme loading. Increasing the amount of glutaraldehyde results in a higher possibility to create additional covalent bonds between enzyme molecules and the carrier. Consequently, higher amounts of glutaraldehyde used led to a smaller fraction of the enzyme molecules that are only physically adsorbed on carrier, see Fig. 2B for the results of the DMSO leaching experiments. As the amount of glutaraldehyde used for modifications increases, activity of immobilized *C. antarctica* lipase B primarily increases, showing the highest value for 0.66% w/w, and subsequently decreases (Fig. 2C). These results indicate that a high concentration of enzyme crosslinker influence the enzyme activity negatively by restricting the enzyme mobility and causing changes in the enzyme conformation, often into a less favorable one, which can result in the deactivation of the enzyme [25]. Furthermore, some undesirable side reactions can occur and connect two copolymers into one and make those copolymers inactive for covalent attachment of enzyme [26].

The same trend is observed for modification of aminated particles with cyanuric chloride (Fig. 3A–C). The maximum value of the activity of Cal-B immobilized on particles activated with cyanuric chloride is reached at 0.050% w/w of cyanuric chloride used for modification. This is much less than in the previous case as the amount of glutaraldehyde for reaching the maximum value of Cal-B activity is 0.66% w/w. Via the DMSO leaching technique, we could show that 91.9% and 48.2% of Cal-B was covalently attached to the macroporous resins activated with cyanuric chloride

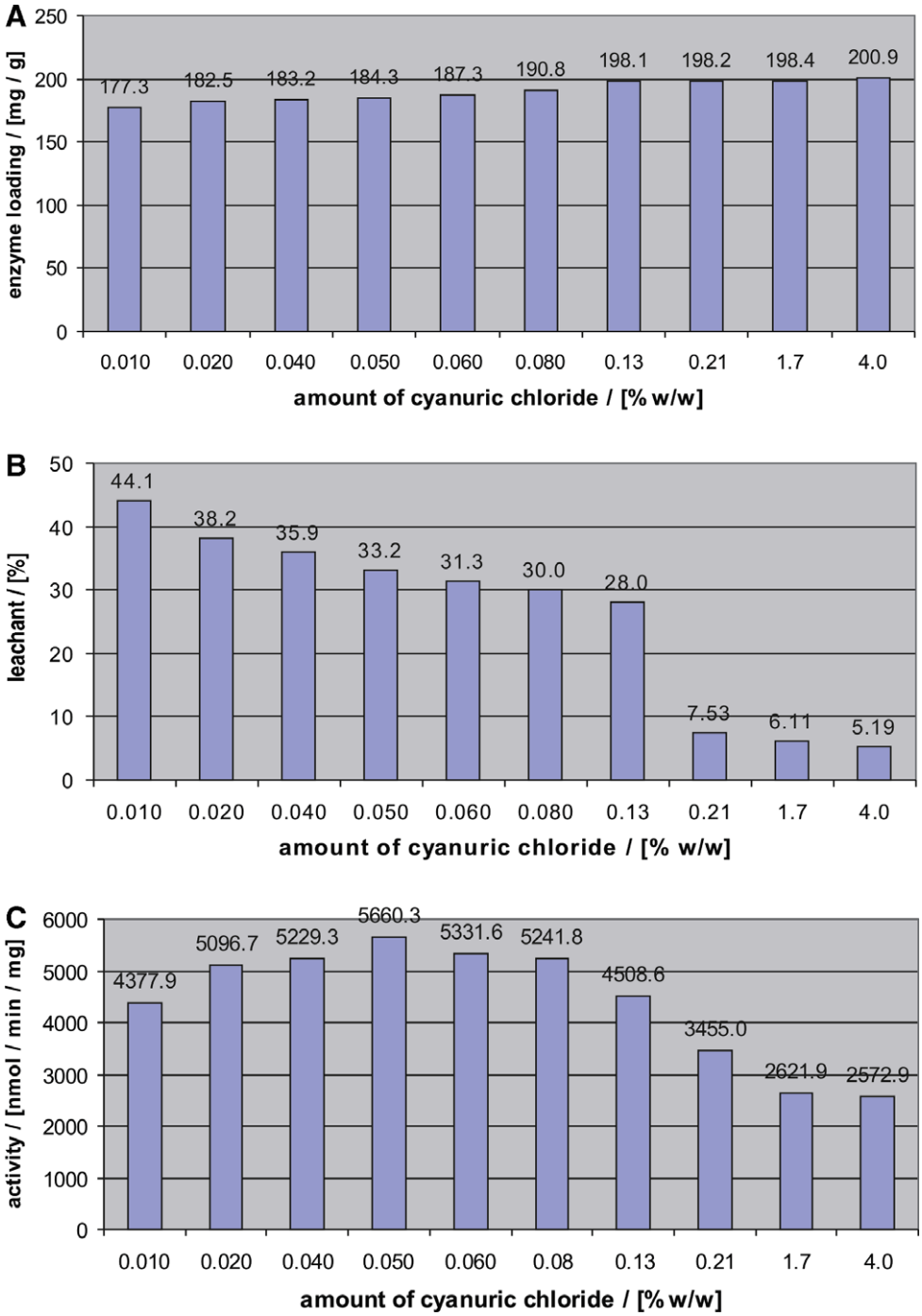

Fig. 3. Influence of the concentration of cyanuric chloride used for activation of aminated copolymer: (a) enzyme loading, (b) leaching and (c) hydrolytic activity.

(using 0.66% w/w for modification) and glutaraldehyde (using 0.050% for modification), respectively. Such high degrees of enzyme crosslinking have a negative impact on protein orientations and access of substrate to active sites is blocked [25]. Therefore, Cal-B immobilized on macroporous particles activated with glutaraldehyde showed a higher hydrolytic activity.

Comparison of the results from the Tables 3 and 4 indicates that activation with glutaraldehyde significantly improved Cal-B activity. For instance, Cal-B immobilized on resin 6 modified with ammonia and subsequently with glutaraldehyde performed much higher activity (7810.1 nmol *p*NP/min/mg Cal-B) than immobilized on resin 6 without modification (4766.0 nmol *p*NP/min/mg Cal-B).

Furthermore, activation with cyanuric chloride pronounced certain improvement of Cal-B activity. Namely, the activity of Cal-B immobilized on resin 5 or 6 modified with various diamines and subsequently with cyanuric chloride is higher than immobilized on corresponding aminated sample.

Comparison of the results from Tables 4 and 5 with hydrolytic activity results for free Cal-B powder (2396.0 nmol *p*NP/min/mg Cal-B) [23] indicates that Cal-B immobilized on modified poly(GMA-*co*-EGDMA) either with glutaraldehyde or cyanuric chloride in most of the cases performs higher activity than free enzyme powder.

4. Conclusions

Candida antarctica lipase B (Cal-B) was immobilized on crosslinked macroporous hydrophilic poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) [abbreviated poly(GMA-*co*-EGDMA)] with good results. Novel carriers for Cal-B immobilization were synthesized by modification of poly(GMA-*co*-EGDMA) with various diamines, 2-fluoroethylamine, glutaraldehyde and cyanuric chloride.

It was shown that the activity of the immobilized Cal-B strongly depends on the binding type between enzyme and matrix beads. Modification of poly(GMA-*co*-EGDMA) with various diamines and 2-fluoroethylamine will support physical connection between enzyme and matrix (that was proven by DMSO leaching experiments). Enzyme loading is much higher for Cal-B immobilized on epoxy-containing copolymers than for Cal-B immobilized on aminated or fluorinated copolymers. These results are attributed to the fact that weak physical adsorption is the dominant connection between Cal-B and aminated and fluorinated copolymers. For the same reason, enzyme activity is not significantly improved by amination or fluorination of poly(GMA-*co*-EGDMA).

Via the DMSO leaching technique, we were able to show that 48.2% and 91.9% of Cal-B was covalently attached to the macroporous resins activated with glutaraldehyde (using 0.66% for modification) and cyanuric chloride (using 0.050% w/w for modification), respectively.

The amount of glutaraldehyde and cyanuric chloride used for modification plays an important role for the enzyme activity. It was shown that increasing the amount of glutaraldehyde or cyanuric chloride used for modification resulted in an increase of the enzyme loading. Consequently, higher amount of glutaraldehyde used led to a smaller fraction of the enzyme molecules that are just physically adsorbed on carrier. As the amount of glutaraldehyde or cyanuric chloride used for modifications increases, activity of immobilized *C. antarctica* lipase B primary increases, showing the highest value for 0.66% and 0.050% w/w, respectively, and subsequently decreases. These results indicate that too high enzyme crosslinker concentrations influence the enzyme activity negatively by restricting enzyme mobility and causing changes in the enzyme conformation.

Furthermore, we were able to prove that Cal-B activity is significantly improved by activation of poly(GMA-*co*-EGDMA) with glutaraldehyde and cyanuric chloride. In most of the cases, Cal-B immobilized on modified epoxy-containing carrier shows a higher activity than free enzyme powder.

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